Biochemical Pharmacology, Vol. 41, No. 10, pp. 1529–1532, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Steroid sulphates inhibit the rat hepatic microsomal glucose-6-phosphatase system

(*Received* 15 *November* 1990; *accepted* 3 *January* 1991)

The liver plays an important role in the regulation of blood glucose levels [1]. At times of stress, or whenever blood glucose levels fall, the liver releases glucose into the bloodstream, which carries the glucose to other tissues which cannot biosynthesize it (e.g. brain) [2, 3]. The two pathways by which glucose is produced in the liver are gluconeogenesis and glycogenolysis. The terminal step of both pathways is catalysed by the same enzyme, microsomal glucose-6-phosphatase (EC 3.1.3.9) [2, 4]. Drugs, genetic deficiencies and disease states which alter glucose-6-phosphatase activity greatly impair the homeostatic regulation of blood glucose levels [4].

Glucose-6-phosphatase is a multi-component system, comprised of (a) the catalytic subunit of the glucose-6-phosphatase enzyme, with its active site situated in the lumen of the endoplasmic reticulum, (b) a regulatory calcium binding protein and (c) at least three transport proteins which allow the substrates glucose-6-phosphate and pyrophosphate, and the products phosphate and glucose, to cross the endoplasmic reticulum membrane (for recent reviews see Refs 4 and 5).

The rate-limiting step in glucose-6-phosphate hydrolysis has been shown to reside in the function of T_1, the microsomal glucose-6-phosphate transport protein [6]. T_1 has a secondary metabolic role, because the glucose-6-phosphate it transports into the lumen of the endoplasmic reticulum is also the substrate for the microsomal glucose-6-phosphate dehydrogenase (G6PD*) [7]. The position of T_1 on the cytoplasmic face of the endoplasmic reticulum membrane makes it a logical site for metabolic regulation, especially in diabetes when both glucose-6-phosphatase activity and hepatic output of glucose are abnormally high [1, 2, 8]. However, there have been very few reports of activation or inhibition of the function of T_1 by endogenous compounds, drugs or their metabolites. Recently, there have been several reports [5, 9, 10] of the presence of inhibitors of T_1 in the livers of some patients with impaired regulation of blood glucose levels. As in all cases only needle biopsy samples were available, it has not been possible to identify the inhibitor or inhibitors involved, but the inhibition could be removed by the addition of bovine serum albumin.

Dehydroepiandrosterone (DHEA) and its sulphate (DHEA-S) are the major adrenal secretory products in man [11], yet their physiological functions have not been fully described. One potentially beneficial effect of DHEA is its ability to prevent the hyperglycaemia associated with experimental diabetes [12]. The mechanism for this effect is unknown, although the well-known inhibition of G6PD by DHEA has been postulated to be involved [13]. DHEA and its sulphate are metabolic substrates for estrogen biosynthesis [14], and it is possible that the anti-diabetic effects of DHEA are mediated via this pathway [14].

We have investigated numerous potential candidate compounds for regulation of T_1, and here we report the inhibition of hepatic microsomal glucose-6-phosphatase activity in intact, but not disrupted, microsomes by two sulphated steroid hormones, DHEA-S and oestrone sulphate (E_1-S), thereby indicating a potential role for these compounds in the physiological regulation of hepatic glucose output.

* Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; DHEA, dehydroepiandrosterone; E_1, oestrone; DHEA-S, dehydroepiandrosterone sulphate; E_1-S, oestrone sulphate.

Materials and Methods

Glucose-6-phosphate (monosodium salt), mannose-6-phosphate (disodium salt), oestrone, dehydroepiandrosterone, oestrone sulphate, dehydroepiandrosterone sulphate, bovine serum albumin (fatty acid and globulin free) and Histone 2AS were purchased from the Sigma Chemical Co. (Poole, U.K.). Sodium cacodylate, also from Sigma, was recrystallized from 95% ethanol [15]. Sodium dodecyl sulphate (specially purified for biochemical work) and tetrasodium pyrophosphate were purchased from BDH Ltd (Poole, U.K.). All other chemicals were obtained from local suppliers, and were of analytical grade or better.

Adult Wistar rats, approximately 12 weeks of age from the colony maintained in the Medical School animal facility, were used throughout. Animals were either allowed free access to food and water, or were denied food for 16 hr prior to being killed. Liver microsomes were prepared from 10% homogenates in 0.25 M sucrose, 5 mM HEPES, pH 7.4 by differential centrifugation. Homogenates were centrifuged at 10,000 *g* for 15 min and the resultant supernatants centrifuged at 105,000 *g* for 1 hr. The microsomal pellets were resuspended in sucrose/HEPES buffer to a protein concentration of approximately 20 mg/mL, aliquoted and stored frozen at −70° until use. All samples were used within 2 months of preparation, and microsome samples were frozen and thawed only once before assay in order to maintain the intactness of the microsomal membrane. Glucose-6-phosphatase activities with glucose-6-phosphate (1 mM) and pyrophosphate (1 mM) as substrates were assayed and calculated according to the method of Burchell *et al.* [16]. All assays were linear with respect to incubation time. DHEA and oestrone (E_1) were dissolved in ethanol, and diluted in cacodylate assay buffer, and DHEA-S and E_1-S were dissolved in cacodylate assay buffer. All steroids were present in the assay mixtures at a final concentration of 100 μM, as this concentration of the steroid sulphates appeared to give substantial inhibition of the glucose-6-phosphatase enzyme system. Inhibition was observed with lower concentrations, but to a lesser extent. It was not possible, however, to use higher concentrations of non-sulphated steroids as a result of solubility problems. The relevant control incubations, in which the solvent only was substituted, were performed for each determination of enzyme activity. Activity was measured in intact and fully disrupted (with Histone 2AS) microsomes [17]. Microsomes isolated from rat liver samples are a mixture of intact and disrupted vesicles. The proportion of intact vesicles in each preparation was quantified by measuring mannose-6-phosphatase activity, which is only expressed in disrupted structures [18]. The intact values in Tables 1 and 2 have been corrected for the contribution by disrupted structures as previously described [16], which eliminates the large errors in activity measurements which occur if even a small proportion of the vesicles are disrupted.

Statistical analysis was performed using Student's *t*-test for paired samples, as described [19].

Protein content was estimated by the method of Peterson [20], on samples which had been frozen and thawed only once.

Results and Discussion

Glucose-6-phosphatase activity in intact microsomal vesicles and *in vivo* is a measure of the combined rate of the glucose-6-phosphatase enzyme and the glucose-6-phosphate, phosphate and glucose transport proteins. To distinguish between the effects of potential inhibitors on each of these proteins it is also necessary to measure (a) glucose-6-phosphatase activity in fully disrupted microsomes, which is a measure of the glucose-6-phosphatase enzyme activity alone, and (b) to use the alternative substrate pyrophosphate in intact microsomes, which is a measure of the combined rates of the glucose-6-phosphatase enzyme and T_2, the phosphate (pyrophosphate) transport protein [6, 16]. Table 1 shows that the inclusion of E_1 and DHEA in glucose-6-phosphate enzyme assays had no effect on the activity of the glucose-6-phosphatase system in intact rat liver microsomal vesicles. The control values given in the legend to Table 1 are lower for fed rat liver microsomes incubated with E_1 and DHEA as a result of the effect of the solvent (ethanol) used to dissolve these compounds (see Materials and Methods). Glucose-6-phosphatase activities were found to be considerably higher in starved rat liver microsomes than those from fed animals (as previously shown [1, 6]), which may account for the fact that the ethanol had little effect on starved rat liver microsomal glucose-6-phosphatase. In contrast, the sulphated forms of both these compounds, E_1-S and DHEA-S, resulted in significant inhibition of glucose-6-phosphatase activity in intact rat liver microsomes from both fed and starved animals. In intact rat liver microsomes, T_1 has been shown to be the rate-limiting step of glucose-6-phosphate hydrolysis [6], suggesting that the inhibition of the glucose-6-phosphatase activity was in fact due to an inhibition of T_1, the glucose-6-phosphate transport protein. This was confirmed by the lack of effect of DHEA-S and E_1-S on the glucose-6-phosphate enzyme activity in rat liver microsomes which had been fully disrupted by treatment with Histone 2AS (data not shown). In all cases, the effects on microsomes prepared from starved rat livers were similar to those from normal, fed rats. DHEA and E_1 also had no effect on the glucose-6-phosphatase activity in disrupted microsomes (not shown). To determine whether these compounds had any effect on T_2 (the phosphate/pyrophosphate transport protein), glucose-6-phosphatase activity was measured in intact rat liver microsomes using pyrophosphate as substrate (Table 2). Again, E_1 and DHEA had no effect on the glucose-6-phosphatase system (data not shown), whereas T_2 was inhibited by both E_1-S and DHEA-S, although the effect of E_1-S on T_2 was much less than on T_1 (Table 1). The inhibition of T_1 and T_2 by DHEA-S was similar (Tables 1 and 2).

Table 1. Effect of steroids and steroid sulphates on glucose-6-phosphatase system activity in intact rat liver microsomes—glucose-6-phosphate as substrate

Additions to assays	Fed	Starved
Control	100	100
E_1	100 ± 5 NS	100 ± 2 NS
E_1-S	67 ± 2*	71 ± 2*
DHEA	100 ± 5 NS	104 ± 4 NS
DHEA-S	74 ± 3*	79 ± 2*

Data are presented as mean ± SEM of averaged duplicate enzyme activity measurements on at least eight separate microsomal preparations, and are expressed as per cent of individual control values. Mean (±SEM) of the control values were: 29 ± 2 (E_1, fed); 41 ± 3 (E_1-S, fed); 29 ± 2 (DHEA, fed); 53 ± 3 (DHEA-S, fed); 71 ± 8 (E_1, starved); 73 ± 9 (E_1-S, starved); 62 ± 4 (DHEA, starved); and 65 ± 7 (DHEA-S, starved); all expressed as nmol/min/mg microsomal protein.

* Significantly different from corresponding control values, $P < 0.001$; NS, not significantly different from control values.

Table 2. Effect of steroid sulphates on glucose-6-phosphatase system activity in intact rat liver microsomes—pyrophosphate as substrate

Additions to assays	Fed	Starved
Control	100	100
E_1-S	85 ± 3*	87 ± 2*
DHEA-S	81 ± 2*	81 ± 2*

Data are presented as mean ± SEM of averaged duplicate enzyme activity measurements on at least five separate microsomal preparations, and are expressed as per cent of individual control values. Mean (±SEM) of the control values were: 48 ± 7 (E_1-S, fed); 55 ± 13 (DHEA-S, fed); 104 ± 14 (E_1-S, starved); and 98 ± 16 (DHEA-S, starved); all expressed as nmol/min/mg microsomal protein.

* Significantly different from corresponding control values, $P < 0.02$.

Table 3. Abolition of steroid sulphate inhibition of fed rat liver glucose-6-phosphatase system by 10 mg/mL bovine serum albumin in intact microsomes

Additions to assays	Control*	Sulphate	Sulphate + BSA
DHEA-S	100	77 ± 4†	106 ± 4 NS
E_1-S	100	65 ± 7†	109 ± 4 NS

Data are presented as mean ± SEM of averaged duplicate enzyme activity measurements on at least three separate microsomal preparations, and are expressed as per cent of individual control values.

* "Control" refers to incubations containing neither sulphate nor BSA, and to which only the buffer used to dissolve the steroid sulphates was added. Mean (±SEM) control values were 77 ± 13 nmol/min/mg microsomal protein (DHEA-S) and 53 ± 2 nmol/min/mg (E_1-S).

† Significantly different from control values, $P < 0.05$; NS, not significantly different from control values.

In vivo inhibition of T_1 in the livers of several patients with abnormal blood glucose regulation was demonstrated to be reversible by the addition of 10 mg/mL bovine serum albumin to glucose-6-phosphatase system assays with intact human liver microsomes [5, 9, 10]. In order to investigate whether the *in vitro* inhibition of T_1 by DHEA-S and E_1-S observed here was also reversible in this manner, assays for glucose-6-phosphatase system inhibition were performed in the presence and absence of 10 mg/mL bovine serum albumin (Table 3). The presence of albumin completely abolished the inhibitory effect of DHEA-S and E_1-S on T_1.

The availability of compounds which inhibit T_1 would have major implications for the therapeutic management of diabetic hyperglycaemia. Recent studies of patients with disorders of glucose regulation showed that T_1 could be inhibited *in vivo*, presumably by an endogenous compound(s), and that this inhibition could be reversed *in vitro* in the presence of high levels of bovine serum albumin [5, 9, 10]. In the present work, we have demonstrated that two common circulating steroids, DHEA-S and E_1-S, are capable of inhibiting T_1 *in vitro*, and interestingly that this inhibition was also reversed by bovine serum albumin. These observations suggest a possible role for sulphated steroids in regulation of the glucose-6-phosphatase system, and therefore regulation of blood glucose levels. DHEA has long been known to possess anti-hyperglycaemic effects in animal models of diabetes [12, 21], although it is not clear whether the active agent is DHEA itself, its sulphate or its potential metabolites via the oestrogenic pathway. The results presented here indicate that one possible mechanism for the anti-hyperglycaemic effect of DHEA is via inhibition of T_1 by DHEA-S and by E_1-S (formed from DHEA or DHEA-S). This is the first report of modulation of T_1 by endogenous compounds.

In conclusion, we have demonstrated for the first time the inhibition of the hepatic microsomal glucose-6-phosphate transport protein by the sulphated form of endogenous steroid hormones. The effect was abolished in the presence of bovine serum albumin, an observation which corresponds exactly with the *in vivo* situation in certain human subjects exhibiting inhibited glucose-6-phosphate transport in the liver. These data have important implications for the potential regulation of blood sugar in normal and disease states by steroid sulphates.

Acknowledgements—This work was supported by grants from The British Diabetic Association, Research Into Ageing and the Scottish Hospitals Endowment Research Trust (to A.B.) and from the Sir Jules Thorn Charitable Trust (to M.W.H.C.). A.B. is a Lister Institute Research Fellow and M.W.H.C. is a Caledonian Research Foundation Research Fellow.

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Biochemical Pharmacology, Vol. 41, No. 10, pp. 1532–1533, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Effect of Ca^{2+}-entry blocker on the stimulation of aerobic metabolism in rats acclimatized to high altitude hypoxia

(*Received* 3 *August* 1990; *accepted* 4 *January* 1991)

Prolonged and repeated exposure to high altitude causes a metabolic acclimatization to hypoxia: aerobic metabolism is enhanced, but anaerobic glycolysis is reduced in rats acclimatized by repeated exposure to a simulated altitude above 5000 m [1, 2]. A higher activity of the mitochondrial oxidative enzyme in the acclimatized rats appears to contribute to the elevated aerobic metabolism [2]. Mitochondrial metabolism can be controlled by Ca^{2+}: Ca^{2+} incorporated into mitochondria activates some dehydrogenases and then enhances oxidative metabolism in mitochondria [3, 4]. In this paper, we analyzed the role of Ca^{2+} in the stimulation of aerobic metabolism of acclimatized rats using a Ca^{2+} entry blocker: Ca^{2+} influx is essential for the stimulation of aerobic metabolism under highly hypoxic conditions.

Materials and Methods

Chemicals and kits for determination of metabolites were purchased as described previously [1, 2]. Diltiazem hydrochloride was a product of the Tanabe Pharmaceutical Co. (Osaka, Japan).

Male Sprague–Dawley rats weighing 120–150 g were acclimatized to hypoxia by repeated exposure to a simulated altitude of 6000 m for 2 hr/day throughout 11 days as described previously [1]. Control groups were not pre-exposed to high altitude. Plasma ketone bodies are markedly lower under fed conditions; hence, both groups were starved overnight before an exposure to a simulated altitude of 8000 m. They were permitted free access to water. Prior to an exposure to the 8000 m altitude, diltiazem solution (2 mg/mL in saline) was injected intraperitoneally into the rats at a dose of 5 mg/kg. An equivalent volume of saline was injected into control animals. Experiments were performed between 1:00 and 4:00 p.m. The decompression rate was 120–150 m/min. The rats remained at the 8000 m-simulated altitude for 1 hr, and returned to sea-level with the same rate. Rats were killed by cervical dislocation at appropriate intervals, and blood was collected in heparinized test tubes. Plasma was immediately separated by centrifugation, and was frozen until analysis.

Plasma lactate, uric acid, and ketone bodies were determined as described previously [1]. Data are expressed as means ± SD.

Results

3-Hydroxybutyrate, a major ketone body in plasma, did not increase when the control rats were exposed to a simulated altitude of 8000 m. However, rats acclimatized by repeated exposure to 6000 m altitude for 11 days showed a marked increase in 3-hydroxybutyrate when exposed to an 8000 m altitude (Fig. 1). Administration of a Ca^{2+}-entry blocker, diltiazem, prior to an exposure to the 8000 m altitude completely blocked the increase in 3-hydroxybutyrate (Fig. 1), and no significant increase in the ketone body was observed in the control and the diltiazem-administered rats. Diltiazem did not show any effect on the level of the ketone body of the control group (data not shown). The ratio of 3-hydroxybutyrate to acetoacetate was 10 and over under the experimental conditions, and, thus, changes in total ketone bodies including 3-hydroxybutyrate plus acetoacetate were essentially identical to those of 3-hydroxybutyrate (data not shown).

Plasma lactate, a marker of glycolytic activity, increased markedly during an exposure of the control rats to an 8000 m simulated altitude. Acclimatized rats showed a

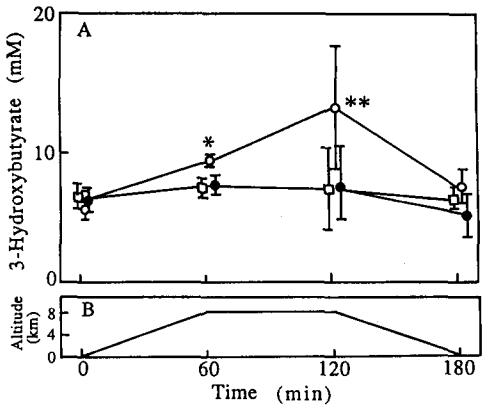

Fig. 1. Effect of diltiazem on the change in plasma concentrations of 3-hydroxybutyrate in rats acclimatized to high altitude hypoxia under conditions of an exposure to an 8000 m simulated altitude. Rats were acclimatized as described in the text. Diltiazem (2 mg/mL) was injected intraperitoneally into the acclimatized rats at a dose of 5 mg/kg prior to submission to an altitude chamber. Blood was collected by decapitation, and plasma ketone bodies were determined. The barometric pressure is represented as the equivalent altitude in panel B. Each point is the mean ± SD; sample size was 4 or 5. Key: (□) control; (○) acclimatized; and (●) diltiazem administered to the acclimatized rats. Asterisks indicate a significant increase in 3-hydroxybutyrate of the acclimatized rats: *$P < 0.001$, and **$P < 0.05$ (Student's *t*-test).